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Notes:

Isolation of amplified DNA sequences from IMR-32 human neuroblastoma cells: Facilitation by fluorescence-activated flow sorting of metaphase chromosomes

(recombinant phage library/gene mapping/amplification and rearrangement)

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ABSTRACT Human neuroblastoma IMR-32 cells have large homogeneously staining regions (HSRs), primarily in the short arms of chromosome 1. We have constructed a recombinant phage library that is enriched for DNA present in the HSR of this chromosome by using fluorescence-activated flow sorting for initial chromosome purification. Eleven distinct cloned DNA segments were identified that showed significantly greater hybridization to IMR-32 genomic DNA, detected by Southern blotting, than to normal human genomic DNA. These sequences have also been localized to the HSR of chromosome 1 by *in situ* hybridization. Based on an approximate 50-fold sequence amplification for each cloned segment and a total HSR size of 150,000 kilobases, the amplified unit in the HSR is estimated to be 3,000 kilobases. Sequences homologous to all cloned HSR DNA segments were mapped to human chromosome 2 by using human–mouse hybrid cells. Further work using *in situ* hybridization demonstrated that cloned HSR segments were localized in the short arm of chromosome 2 in both normal and IMR-32 cells. Thus, the amplification of these sequences in IMR-32 cells may have involved transposition from chromosome 2 to chromosome 1.

A number of human tumors (1–3), in particular neuroblastomas (4–6), exhibit homogeneously staining chromosomal regions (HSRs) and extrachromosomal double minute objects (DMs) which are probably cytological manifestations of amplified genes (see ref. 7 for a review). These HSRs and DMs are often observed in tumor tissue removed prior to institution of therapy, and it seems reasonable to suggest that they may contain genetic information responsible for some of the phenotypic properties of the tumor cells. This hypothesis can be tested by isolation and characterization of DNA within the amplified chromosomal regions. In one study (8, 9), DMs of a rat adrenal tumor cell line were isolated by differential centrifugation and cloned in a λ bacteriophage. We have utilized a different approach to isolate sequences specific for the HSRs of a human neuroblastoma cell, exploiting the increased size imparted to metaphase chromosomes by these HSRs.

The human neuroblastoma cell line IMR-32, which was established from tissue of a patient who had not been exposed to any chemotherapy (10), constitutes an excellent system for analysis of HSR-specific sequences. IMR-32 cells, which exhibit some karyotypic heterogeneity, possess, in addition to a normal chromosome 1, two abnormal chromosomes 1 in which an HSR is inserted in the short arm (in the middle of band 1p3). The HSR-containing chromosome 1 is 50% larger than the normal

chromosome 1 (11) and hence is easily separable from it and all other, smaller, human chromosomes by fluorescence-activated sorting of isolated IMR-32 metaphase chromosomes after they have been stained with a DNA-specific fluorochrome such as 33258 Hoechst (12).

From the DNA of a few million metaphase chromosomes enriched for the HSR-containing chromosome 1 by flow sorting, we have prepared recombinant libraries in the Charon phage 21A (13) from which cloned sequences specific for the HSR have been identified both by DNA blotting (14) and by *in situ* hybridization (15, 16). These cloned sequences provide a unique opportunity to study the composition, formation, and functional significance of the amplified sequences in this and other neuroblastoma cell lines.

MATERIAL AND METHODS

IMR-32 neuroblastoma cells, originally obtained from the American Type Tissue Culture Collection and maintained in culture for several months, were used in these experiments. Cells were cultured in Ham F10 medium supplemented with 10% fetal calf serum. Cytological metaphase chromosome preparations were stained with quinacrine or chromomycin A3 plus methyl green as described (17).

Nonconfluent cells were trapped at metaphase by treatment with Colcemid (45 ng/ml) for 20 hr. Cells were detached by shaking, decanted, and washed with Hanks buffered salt solution. The cells were resuspended at a concentration of approximately 2×10^7 /ml in 7.5 mM Tris·HCl/7.5 mM MgCl₂, pH 7.5 (18) for 15 min, after which Triton X-100 was added to a concentration of 1% and the cells were incubated for another 10 min at room temperature and then placed on ice. Cells were disrupted by three or four separate 20-sec shearing pulses in a VirTis 60 homogenizer at 5,000 rpm, diluted with 2 vol of 20 mM Tris/40 mM MgCl₂/120 mM NaCl, stained with 33258 Hoechst (4 μ g/ml), and analyzed in a FACS II flow cytometer using excitation at 351–364 nm (19). Chromosomes were stored at -70°C before DNA isolation.

DNA was prepared from 5×10^6 chromosomes that had been pelleted at 10,000 rpm ($16,000 \times g$) for 30 min, resuspended in 100 mM NaCl/50 mM Tris, pH 7.5/1 mM EDTA/0.1% NaDodSO₄ containing tRNA (25 μ g/ml) and proteinase K (250 μ g/ml), and incubated at 37°C for 2 hr. The digest was then extracted twice with phenol/chloroform/isoamyl alcohol, 24:1:1

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Abbreviations: HSR, homogeneously staining region; DMs, double minute objects; NaCl/Cit, standard saline citrate; kb, kilobase(s).

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(vol/vol), twice with chloroform, and once with diethyl ether. The DNA was subjected to ethanol precipitation and resuspended in 20 μ l of 10 mM Tris (pH 7.5); 4.5 μ l of this DNA preparation (approximately 135 ng) was digested to completion with *Hind*III.

Approximately 35 ng of chromosomal DNA digested with *Hind*III was ligated with 100 ng of *Hind*III-digested and alkaline phosphatase-treated Charon 21A phage arms by using T4 DNA ligase, and the ligated DNA was packaged according to the procedure of Blattner *et al.* (13). In two such experiments, using a total of approximately 70 ng of chromosomal DNA, 60,000 plaque-forming units were obtained. These phages were amplified on lawns of LE 392 bacteria and purified by centrifugation in a cesium chloride gradient.

Primary screening of the library for phage containing repetitive human DNA was carried out by the method of Benton and Davis (20). Human placental DNA labeled with 32 P by nick-translation (21) was used as the probe. Of the phage plaques that showed negative hybridization to the probe, 120 were picked out by glass pipet and eluted into 0.5 ml of 0.01 M Tris-HCl, pH 7.2/0.01 M MgCl₂/0.1 M NaCl. Conditions for hybridization and detection of 32 P-labeled DNA to phage or DNA immobilized on nitrocellulose filters as well as the procedure for isolation of phage DNA were as described (19).

DNA was further analyzed by digestion with *Hind*III, electrophoresis in agarose gels, and blotting onto nitrocellulose (14). Of the 120 phage DNA samples tested, 66 did not hybridize to total human DNA. DNA from each of these 66 phage was digested with *Hind*III, labeled with 32 P by using T4 DNA polymerase [ref. 22 with minor modification (19)]. The labeled inserts were then hybridized to nitrocellulose imprints of *Hind*III-digested IMR-32 or 46,XY lymphoblast DNA that had previously been subjected to agarose gel electrophoresis (3 μ g of DNA per lane). DNA concentrations were measured with the dye 4',6-diamidinophenylindole (DAPI) (23).

Phage inserts that appeared to be amplified in IMR-32 DNA, in comparison with 46,XY lymphoblast DNA, were subcloned in the plasmid pBR322 which was amplified by transforming *Escherichia coli* MC1061 (24). Plasmid DNA was purified by centrifugation in cesium chloride gradients containing ethidium bromide (25).

For *in situ* hybridization (15, 16), air-dried slides of metaphase cells, stored at least 1 week in a vacuum desiccator, were denatured in a mixture containing 70% deionized formamide and 2 \times standard saline citrate (NaCl/Cit; 1 \times NaCl/Cit is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) at 65°C for 2 min and then dehydrated through 70%, 80%, and 95% ethanol. Plasmids with HSR segments were labeled, by nick-translation, to 2–4 $\times 10^7$ cpm/ μ g with [3 H]dTTP, [3 H]dCTP, and [3 H]dATP. The labeled probes were denatured in hybridization mixture (50% formamide, 2 \times NaCl/Cit, 10% dextran sulfate, and sheared single-stranded DNA at 100 μ g/ml) at 70°C for 5 min and then cooled in ice. The approximate size of the single-stranded probe was 500–1,500 bases. Approximately 2.5 $\times 10^5$ cpm of probe (≤ 15 ng) in 50–60 μ l of hybridization mixture was added per slide, a large coverslip was applied, and the slides were incubated in a humid atmosphere at 42°C for 20 hr. After the annealing, the coverslip was removed and the slides were washed at 39°C first with 50% deionized formamide in 2 \times NaCl/Cit and then with 2 \times NaCl/Cit alone (or five times with 2 \times NaCl/Cit at 39°C) and finally dried through an ethanol series before being dipped in Kodak NTB-2 emulsion, exposed, and developed.

Somatic cell hybrids between human and mouse or Chinese hamster cells were prepared and characterized for chromosome content by cytological and enzymatic analyses as described (26).

RESULTS

The IMR-32 cells used for the present experiments contain a modal number of 48 chromosomes. Approximately 70% of cells have only one normal chromosome 1 plus two apparently identical abnormal chromosomes 1 (Fig. 1). These abnormal chromosomes result from insertion of a segment of homogeneously staining material into region 1p3, which is clearly defined by reverse banding with chromomycin and methyl green (Fig. 1B) and is early replicating (27). In addition, some cells contained a third abnormal, medium-to-large-size chromosome of variable morphology which often but not invariably exhibited a segment staining homogeneously with quinacrine or chromomycin A3/methyl green. Evidence that this chromosome contained amplified sequences also found in the HSRs of the abnormal chromosomes 1 came primarily from *in situ* hybridization (see below). Other karyotypic variabilities existed in these cells; most frequently these were an extra copy of chromosome 12, only a single copy of chromosome 16, an E-group-size marker chromosome, and an increase in the size of the short arm of the normal chromosome 1.

The large size of the HSR-containing chromosome 1 produced a unique peak in the chromosome flow histogram (Fig. 2) after staining with 33258 Hoechst and therefore permitted its separation from the remaining chromosomes by fluorescence-activated flow sorting. Based on microscopic examination, approximately half of the objects sorted from this peak were the HSR-containing chromosome 1; this impression was reinforced by subsequent analysis of cloned DNA segments isolated from sorted chromosomes. The remaining sorted material probably consisted primarily of aggregated small chromosomal material as well as subnuclear fragments and a few large chromosomes.

Approximately 10^7 chromosomes were sorted from the peak enriched for HSR-containing chromosomes; DNA isolated from 5×10^6 of these sorted chromosomes totaled 0.5 μ g. Of this, two samples, totaling approximately 70 ng, were cleaved with *Hind*III and cloned in the λ phage Charon 21A. The two resulting libraries of recombinant phage, totaling approximately 60,000 plaque-forming units, were used for all subsequent experiments described. These libraries contained an average of

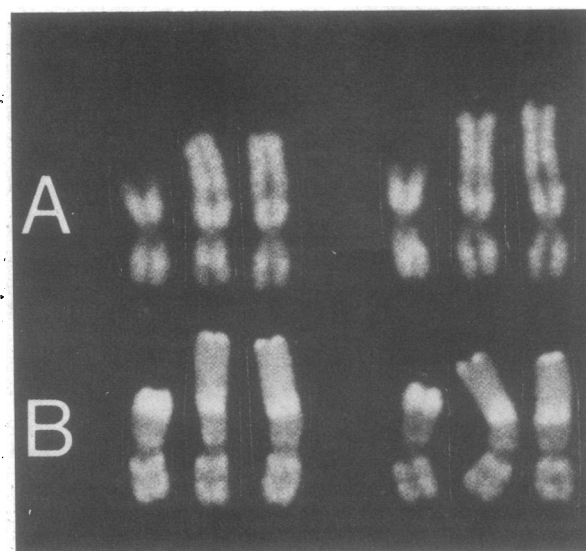


FIG. 1. Chromosomes 1 seen in IMR-32 cells. The normal chromosome 1 is at the left and the HSR-containing chromosomes 1 are at the right of each group of three chromosomes. (A) Quinacrine banding; (B) reverse banding with chromomycin A3/methyl green.

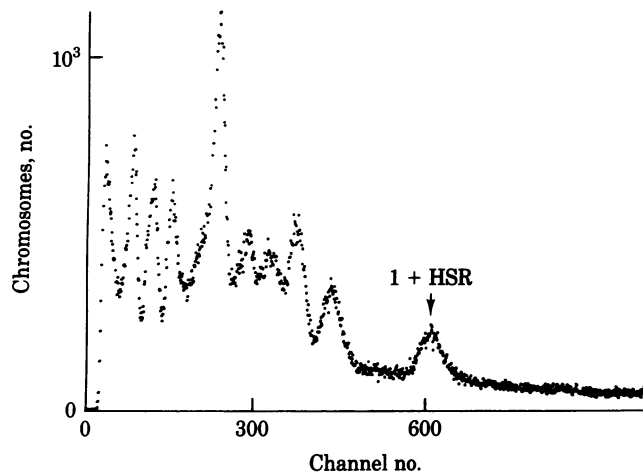


FIG. 2. Flow histogram obtained from FACS II showing chromosome distribution of IMR-32 cells. Approximately 10 distinct peaks were obtained. The arrow indicates the peak (chromosome 1 + HSR) that was collected for isolation and cloning of HSR DNA.

95% recombinant phage, based on Benton–Davis (20) plaque and Southern (14) blot hybridizations. Of these, 10% lacked highly repeated sequences [mean size, 3–4 kilobases (kb)] and hence could be tested for their amplification in IMR-32 genomic DNA in comparison with normal genomic DNA.

Cloned DNA segments lacking highly repeated human sequences were labeled with ^{32}P and used to probe Southern blots of IMR-32 or 46,XY DNA that had been digested to completion with *Hind*III. Sequences presumptively assigned to amplified chromosomal regions were those that hybridized significantly more intensely to the IMR-32 DNA (Fig. 3A). Thirteen of 66 DNA segments tested exhibited such hybridization (Table 1). Subsequent Southern blots, comparing hybridization of these probes to 3 μg of 46,XY DNA, and increasing dilutions of IMR-32 DNA mixed with salmon sperm DNA (digested with *Hind*III to a total of 3 μg per lane) were used to determine that IMR-32 cells contained approximately 50 times the representation of those sequences as did 46,XY cells (data not shown). Most of the remaining probes hybridized with identical intensity to fragments of indistinguishable size in *Hind*III digests of 46,XY and IMR-32 DNA (Fig. 3B). One cloned DNA segment (no. 3

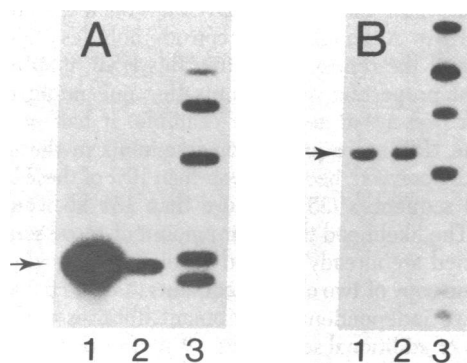


FIG. 3. Detection of sequence amplification in IMR-32 cell DNA. Autoradiographs of nitrocellulose blots (14) in which ^{32}P -labeled cloned DNA obtained from the sorted chromosome library were hybridized to 3 μg of IMR-32 DNA (lane 1) or 46,XY lymphoblast DNA (lane 2). Lane 3 contains a *Hind*III digest of ^{32}P -labeled phage λ DNA for size markers. (A) A 1.85-kb fragment which shows enhanced hybridization to IMR-32 DNA and presumably derives from the HSR region; (B) a 4.5-kb fragment which shows equal hybridization to IMR-32 and 46,XY DNA and therefore does not derive from an amplified region.

Table 1. Cloned DNA segments localized to the HSRs of IMR-32 cells

No.	Size, kb	Localized to HSR by <i>in situ</i> hybridization	Presumptive mapping to normal human chromosome 2*
1	6.7 [†]	+ [‡]	+ [§]
2	6.3 ^{†,¶}		+
3	4.3 ^{*,¶,**}	+	+ [§]
4	2.2 ^{†,**}		+
5	2.15	+	+
6	1.85	+	+ [§]
7	1.80	+	+
8	1.75	+	+
9	1.70		+
10	1.65	+	+ [§]
11	0.46	+	+ [§]

* Localization by hybrid DNA panel (see Fig. 5).

[†] These inserts contained a small amount of repeated sequences. The other DNA segments have not yet been examined.

[‡] *In situ* hybridization was performed with a 1.7-kb subclone that lacked repeated DNA sequences.

[§] Localization confirmed by *in situ* hybridization (see Fig. 6).

[¶] These two inserts were isolated from each of two phage libraries.

^{||} Repeated sequences apparent in *in situ* hybridization, obscuring localization to HSR.

** This DNA segment hybridized weakly to a 8.5-kb fragment of *Hind*III-digested IMR-32 genomic DNA.

in Table 1) hybridized to two *Hind*III fragments in IMR-32 DNA (with different distributions of internal and surrounding restriction enzyme digestion sites) but not in any of the 46,XY DNA samples tested.

Southern blots of agarose gels containing in each lane 0.2 μg of every DNA segment presumptively assigned to the HSRs of IMR-32 cells and subcloned in the plasmid pBR322 (28) were then probed with ^{32}P -labeled DNA from each plasmid to determine that two inserts (6.3 and 4.3 kb, respectively) were present twice in the original collection of 13; the remaining 9 inserts hybridized only with each other.

As an additional test of their chromosome location in IMR-32 cells, nine of the cloned inserts were labeled with ^3H -labeled nucleotides and hybridized *in situ* (15, 16) to cytological preparations of mitotic IMR-32 cells. For all probes tested (Table 1), extensive hybridization was observed throughout the distal part of the long arm of the two largest chromosomes of the cell, exactly as expected if these sequences were amplified and present in the HSR located in region p3 of chromosome 1 (Fig. 4A). In approximately 30% of cells, extensive hybridiza-

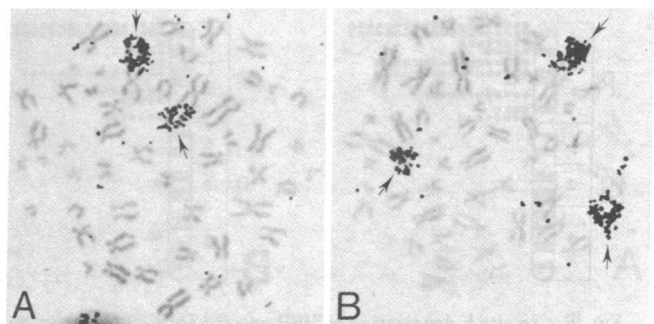
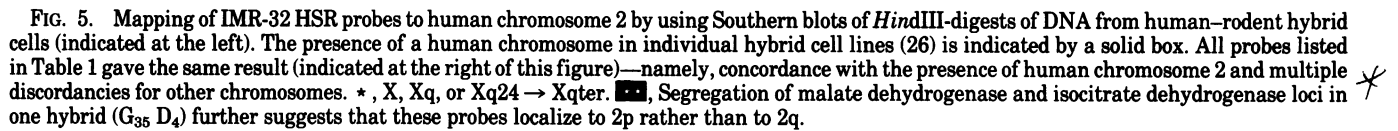


FIG. 4. *In situ* hybridization of a cloned, amplified, 4.3-kb segment to metaphase chromosomes of IMR-32 cells. Extensive hybridization of this probe, no. 3 of Table 1, was observed over the elongated HSR-containing arm of chromosome 1 (A). In addition, about 30% of cells showed a third site of intense hybridization (B) which was on an additional derived chromosome present in these cells.



All the sequences examined localized to human chromosome 2, rather than to 1, when a human-rodent hybrid panel was used (Fig. 5). These observations were further confirmed in some HSR clones (Fig. 6) by *in situ* hybridization in both normal and IMR-32 cells, revealing nonamplified hybridization to the short arm of chromosome 2.

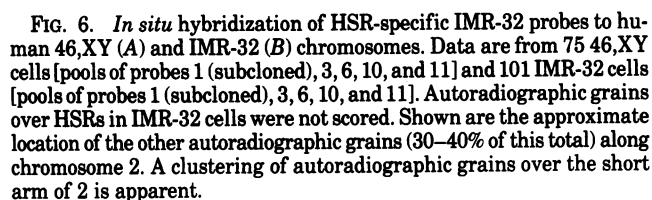
The experiments described here outline a successful strategy for isolating and cloning of DNA from amplified chromosomal regions by using fluorescence-activated flow sorting to enrich for metaphase chromosomes containing HSRs. In the present experiments, one-fifth of all inserts screened from the recombinant phage libraries produced were localized to the amplified chromosomal segments. Had the sorted chromosomes consisted only of the HSR-containing chromosome 1, one-third of the DNA would have been located in the amplified region. By this comparison, the sorted chromosomes were approximately 60% pure. Because the HSRs of IMR-32 cells contain 4–5% of the total cellular DNA, the enrichment for DNA in this region obtained by chromosome sorting was 4- to 5-fold. Although this enrichment is modest compared with that possible by flow sorting of a small chromosome—e.g., chromosome 21 or 22 (29)—which constitutes only 2% of cellular DNA, it still greatly facilitated subsequent screening of the cloned DNA inserts. In

Based on microphotometric data of Balaban-Malenbaum *et al.* (11) indicating that the HSR of IMR-32 contains an amount of DNA corresponding to 1.5×10^5 kb and with an assumed approximate repetition frequency for the HSR-specific cloned segments of 50 per haploid genome, the average repeat unit within the HSR can be computed to be 3×10^3 kb—i.e., the typical size of a band in moderately extended prometaphase (32) chromosomes. Although the mechanism of the amplification leading to the HSR is unknown, data obtained by two different methods indicate that the original sequences are present in normal cells on chromosome 2, and that they remain on chromosome 2 in IMR-32 cells. That is, the amplification of this sequence is somehow associated with its transposition to chromosome 1, a chromosome that exhibits an unusual degree of structural aberrations in neuroblastoma cells (33, 34). The observation, in 30% of cells, of a third chromosome containing amplified sequences common to the HSRs attests to the potential fluidity of that part of the IMR-32 genome containing these sequences.

Thus far, more than 40 kb of DNA has been cloned from the IMR-32 HSR. Although this is considerably less than the average size of the repeat unit within this HSR, it still reflects a significant proportion of segments that one might expect by using our present strategy. For example, if half of the DNA inside the HSR were present on fragments in the size range 0.5–6.7 kb observed, because fewer than 10% of these lack highly repeated sequences (35) no more than 150 kb would be expected. The likelihood that a fair amount of those sequences to be obtained are already isolated is reinforced by the observation of one copy of two cloned segments (4.3 and 6.3 kb) in each of the two independent recombinant libraries screened. Acquisition of additional sequences within the HSR would be improved by the use of cloning vectors accepting different or larger DNA inserts.

In addition to their obvious usefulness for studies of DNA amplification, the cloned DNA segments isolated from the HSRs of IMR-32 cells may prove of value in understanding the role of amplification of these sequences in the biological phenotype of human neuroblastoma cells which often overproduce one or more enzymes associated with catecholamine metabolism (36). DNA from IMR-32 cells has weak activity at best (37) when tested

* Correction - Proc. Natl. Acad. Sci. USA - Vol 80 (1983) p 7025



quest that the following correction be noted. All hybrids described in Fig. 5 contained at least part of the human X chromosome, and the box for each hybrid in the column corresponding to the human X should have been darkened. This clerical error does not alter any conclusions in the paper.

for its ability to transform mouse (NIH/3T3) cells, and an "oncogene" highly effective at such transformation, as isolated from SKN-SH neuroblastoma cells, is not amplified in IMR-32 cells (38). Additional experiments (data not shown) have determined that dihydrofolate reductase sequences are not amplified in IMR-32 cells.

The potential functional significance of the DNA in the HSR of IMR-32 cells nevertheless is suggested by the observation that its DNA replicates early in S phase (27) and by preliminary data indicating that a few sequences tested thus far hybridize strongly on blots (39) of total cellular IMR-32 poly(A)⁺ RNA, consistent with their transcription. It will be important to characterize the HSR-specific sequences transcribed in IMR-32 cells and, if possible, their protein products as well. Similarly, it will be essential to examine the homology between DNA cloned from the HSR of IMR-32 cells and that amplified in other neuroblastoma cells. Preliminary results indicate that at least one of the sequences amplified in IMR-32 cells is also amplified in three other neuroblastoma cell lines which, unlike our IMR-32 cells, contain DMs, suggesting that these sequences may be related to the neuroblastoma phenotype.

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